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Synthesis of Haptens and Potential Radioligands and Development of Antibodies to Insect Growth Regulators Diflubenzuron and BAY SIR 8514

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16. ABSTRACT

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Synthesis of Haptens and Potential Radioligands and Development of Antibodies to Insect Growth Regulators Diflubenzuron and BAY SIR 8514

Siong I. Wie, Alan P. Sylwester, Keith D. Wing, and Bruce D. Hammock*

A variety of synthetic approaches were undertaken, leading to potential haptens and radioligands for the benzoylphenylurea insect growth regulators diflubenzuron and BAY SIR 8514. One successful approach involved derivatization of the aniline nitrogen by ethyl 4-bromobutyrate followed by reaction with an appropriate isocyanate and cleavage of the ethyl ester to yield a free carboxypropyl "handle". Useful haptens were also synthesized by using a 3′-phenolic metabolite of diflubenzuron as well as acetate and amine functionalities in the 4′ position while the *N*-sulfenyl bond proved too unstable for use as an antigen. With the exception of the sulfenylated derivatives, the haptens lacked significant biological activity on three insect species. Following protein coupling by the active ester or water-soluble diimide method, antibodies were raised to two diflubenzuron haptens in each of seven rabbits immunized as demonstrated by radioimmunoassay using [^{14}C]diflubenzuron, Ouchterlony gel diffusion, and immunoelectrophoresis.

The insect growth regulators (IGRs) diflubenzuron [Dimilin, TH-6040, *N*-[[(4-chlorophenyl)amino]-carbonyl]-2,6-difluorobenzamide, 1] and BAY SIR 8514 [*N*-[[[4-(trifluoromethoxy)phenyl]amino]carbonyl]-2-chlorobenzamide, 18] are promising insect control agents. Since these compounds and structurally related IGRs are either in use or projected for commercial use, information regarding their environmental fate is exceedingly important. Numerous studies have been carried out on the persistence and routes of degradation of benzoylphenylurea IGRs under a variety of conditions [see Maas et al. (1980) and Hammock and Quistad (1981) for references]. The classical methods of diflubenzuron analysis by GLC or HPLC are of adequate sensitivity and reproducibility; however, these methods are very expensive and tedious (Corley et al., 1974; Oehler and Holman, 1975; Lawrence and Sundaram, 1976; Maini and Deseo, 1976; Schaefer and Dupras, 1977; Worobey and Webster, 1977, 1978; DeMilo et al., 1978; DiPrima et al., 1978; Rabenort et al., 1978; Monem and Mumma, 1981). So that a large number of samples can be inexpensively analyzed within a short period of time, improved analytical methods are needed. As shown by previous reports on the development of pesticide-specific antibodies, immunochemical procedures offer some advantages over GLC- and HPLC-based methods (Hammock and Mumma, 1980). Therefore a study was undertaken to develop simple immunoassays for the benzoylphenylurea IGRs. This report illustrates the synthesis of diflubenzuron and BAY SIR 8514 protein conjugates and their use in the induction of IGR-specific antibodies.

MATERIALS AND METHODS

Analytical TLC was performed on silica gel F_{254} plates (EM Laboratories), while preparative TLC was done on 2000-μm silica gel plates with fluorescent indicators (Analtech). Compounds were detected by their quenching of gel fluorescence when viewed under shortwave ultraviolet (UV) light (254 nm). TLC systems found useful for this study include toluene–propanol (10:1), toluene–propanol–acetic acid (10:1:0.1), toluene–propanol–NH_4OH (10:1:0.1), hexane–ethyl acetate (3:2), and hexane–ether (5:1). Each stable intermediate and final synthetic product showed only a single spot on TLC in a minimum of three solvent systems including at least one acidic and one basic system. Proton magnetic resonance (^{1}H NMR) spectra were obtained on a Varian EM-390 in a dilute solution of the indicated solvent with or without deuterium oxide and always by using tetramethylsilane as the internal standard. Infrared (IR) spectra were run as thin films of the respective compound on silver chloride plates or in Nujol with Beckman Model 4240 or Perkin-Elmer Model 700-A spectrophotometers. All reactions were carried out under N_2 in subdued light using dry, freshly distilled solvents. Water was doubly distilled except in the case of saturated brine. An overview of synthetic procedures is shown in Figure 1.

Synthesis. *2,6-Difluorobenzoyl Isocyanate.* To a magnetically stirred suspension of 2,6-difluorobenzamide (98%, 0.075 mol) in 65 mL of dry 1,2-dichloroethane was added freshly distilled oxalyl chloride (0.097 mol). The mixture was then refluxed for 24 h and the solvent distilled off at reduced pressure and the remaining syrup vacuum distilled to give 11.25 g of clear oil in 90% yield: bp 62–63 °C at 0.4 mmHg (bath temperature 90–95 °C); NMR (CCl_4) δ 7.00 (t, 2) and 7.50 (m, 1) and the absence of amine proton signals; IR (neat, AgCl) 2252 cm^{-1} (s) and the absence of N–H stretch band. 2-Chlorobenzoyl isocyanate was prepared in a similar manner as earlier described by Wellinga et al. (1973a) for 2,6-dichlorobenzoyl isocyanate as shown in Figure 1.

N-[(*Ethoxycarbonyl*)*methyl*]-*4-chloroaniline* (*B*, *Figure 2*). A suspension of 4-chloroaniline (0.02 mol), ethyl bromoacetate (0.02 mol), and sodium acetate (0.02 mol) in 5 mL of ethanol was heated to reflux for 8 h. The reaction mixture was poured into 150 mL of water and extracted with ether (2 × 50 mL). The ether layer was

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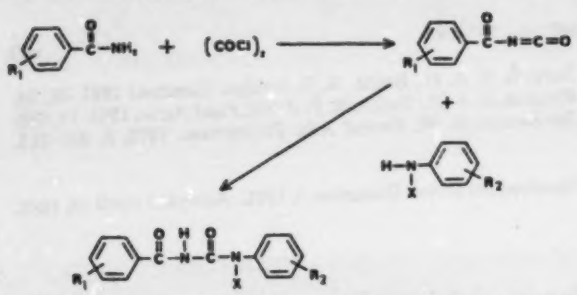

Figure 1. Generalized route for the synthesis of potential haptens for the benzoylphenylurea insect growth regulators. The appropriate benzamide is converted to an isocyanate by exposure to oxalyl chloride. The isocyanate is condensed with a substituted aniline to yield the final product. R_1, R_2, and X are described in Figure 3 as compounds 1–13 and 18–27.

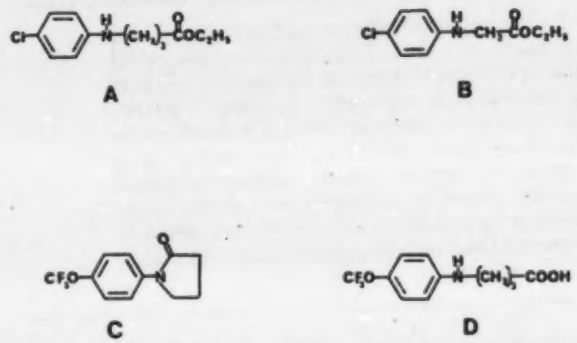

Figure 2. Intermediates used in the synthesis of several haptens for benzoylphenylurea IGRs. (A) *N*-[(Ethoxycarbonyl)propyl]-4-chloroaniline; (B) *N*-[(ethoxycarbonyl)methyl]-4-chloroaniline; (C) *N*-[(4-trifluoromethoxy)phenyl]-γ-lactam; (D) *N*-(carboxypropyl)-4-(trifluoromethoxy)aniline.

washed with water and brine and filtered through a cone of anhydrous sodium sulfate. The ether was removed under reduced pressure and the residual crystals were recrystallized from 90% ethanol to give 3.4 g, an 80% yield of light platelets: mp 92–94 °C; NMR (CCl_4) δ 1.23 (t, 3, CH_3), 3.75 (s, 2, CH_2N), 4.20 (br s, 1, NH, lost with D_2O), 4.15 (q, 2, OCH_2), 6.42 (d, 2, Ph), 7.03 (d, 2, Ph). *N*-[(Ethoxycarbonyl)methyl]-4-(trifluoromethoxy)aniline was prepared similarly.

N-[3-(*Ethoxycarbonyl*)*propyl*]-*4-chloroaniline* (*A, Figure 2*). A suspension of 4-chloroaniline (0.02 mol), ethyl 4-bromobutyrate (0.02 mol), and sodium acetate (0.02 mol) was heated to reflux in 5 mL of ethanol for 12 h. Similar workup and recrystallization from ethyl acetate–hexane gave a 65% yield of platelets: mp 61–62 °C; NMR (CCl_4) δ 1.23 (t, 3, CH_3), 1.87 (quint, 2, CCH_2C), 2.32 (t, 2, CH_2CO), 3.09 (t, 2, NCH_2), 3.55 (br s, 1, NH), 4.05 (q, 2, OCH_2), 6.40 (d, 2, Ph), 7.02 (d, 2, Ph); IR (CCl_4, AgCl, thin film) 3510 (N–H stretch), 1730 cm^{-1} (C=O stretch ester). Traces of the disubstituted product and *N*-ethyl-4-chloroaniline were isolated, but 30% of the remaining aniline could be accounted for as *N*-(4-chlorophenyl)-γ-lactam: NMR (CCl_4) δ 2.15 (quint, 2, CCH_2C), 2.50 (t, 2, CH_2CO), 3.75 (t, 2, NCH_2), 7.25 (d, 2, Ph), 7.60 (d, 2, Ph); IR ($CHCl_3$, AgCl, thin film) 1681 cm^{-1} (C=O stretch). The γ-lactam could be hydrolyzed and reesterified in 75% yield following recrystallization as described for the *N*-[(4-trifluoromethoxy)phenyl]-γ-lactam below (C, Figure 2). The resulting product was then used in subsequent syntheses. *N*-[3-(Ethoxycarbonyl)propyl]-4-(trifluoromethoxy)aniline was prepared in a similar manner: NMR ($CDCl_3$) δ 1.22 (t, 3, CH_3), 1.85 (quint, 2, CCH_2C), 2.32 (t, 2, CH_2CO), 3.25 (t, 2, CH_2N), 4.10 (q, 2, OCH_2), 6.60 (d, 2, Ph), 7.05 (d, 2, Ph).

Hydrolysis of N-[4-(Trifluoromethoxy)phenyl]-γ-lactam. The γ-lactam (C, Figure 2) (0.013 mol) was dissolved in 10 mL of ethanol and 20 mL of 20% NaOH in water was added. The mixture was brought to reflux for 18 h when TLC showed no lactam and a lower R_f product. The reaction mixture was poured into 100 mL of water and extracted sequentially with hexane and ether (50 mL, 1×) to remove trace, high R_f impurities. The aqueous phase was acidified to pH 2 with HCl and extracted with ether (100 mL, 2×). The ether extract was washed with water (25 mL, 2×), shaken with sodium sulfate, and filtered through magnesium sulfate. Removal of the solvent afforded 3 g of brownish crystals that were washed with CCl_4 to yield colorless crystals, mp 86–88 °C, in 75% yield (D, Figure 2): NMR ($CDCl_3$) δ 1.90 (quint, 2, CCH_2C), 2.45 (t, 2, CH_2CO), 3.15 (t, 2, CH_2N), 6.50 (d, 2, Ph), 7.00 (d, 2, Ph), 7.30 (s, 2, NH, COOH), 7.30 lost with D_2O; IR ($CHCl_3$, thin film, AgCl) 3600–2800 (br s), 1700 (s), 1500 (m), 1275 (s), 1160 cm^{-1} (m).

N-[[(4-Chlorophenyl)[3-(ethoxycarbonyl)propyl]-amino]carbonyl]-2,6-difluorobenzamide (3). 2,6-Difluorobenzoyl isocyanate (1.3 mmol) was added via syringe to a stirred solution of *N*-[3-(ethoxycarbonyl)propyl]-4-chloroaniline (A) in 5 mL of dry benzene. The mixture was stirred for 12 h at room temperature, the benzene removed under reduced pressure, and the residue recrystallized from ethyl acetate–hexane to yield white crystals (mp 92–94 °C) in 85% yield: NMR ($CDCl_3$) δ 1.18 (t, 3, CH_3), 1.58 (s, 1, NH), 1.80 (quint, 2, CCH_2C), 2.25 (t, 2, CH_2CO), 3.63 (t, 2, CH_2N), 4.03 (q, 2, CH_2O), 6.90 (t, 2, Ph), 7.30 (m, 5, Ph). The *N*-(ethoxycarbonyl)methyl derivative of diflubenzuron (2) and the *N*-(ethoxycarbonyl)propyl derivative of BAY SIR 8514 (19) were prepared by analogous procedures. The *N*-(ethoxycarbonyl)propyl derivatives of diflubenzuron (3) and BAY SIR 8514 (19) were hydrolyzed at room temperature to the corresponding free acids (*N*-carboxypropyl derivatives 4 and 20) in quantitative yield. The structures were confirmed by NMR and IR and by reesterifying a small amount of the acids with diazoethane and finding the spectra and chromatographic behavior of the products indistinguishable from those of the original esters.

N-Propargyl-4-(trifluoromethoxy)aniline. Freshly distilled propargyl bromide (0.04 mol) was added dropwise to a refluxing solution of 4-(trifluoromethoxy)aniline (0.08 mol) in 30 mL of ethanol. The mixture was refluxed for 2 h, cooled to room temperature, poured into 200 mL of acidic ice water, and extracted with ether. The ether layer was dried with sodium sulfate and chromatographed on Florisil to yield monosubstituted aniline (65%), disubstituted aniline (22%), and recovered starting aniline. *Monosubstituted* aniline: NMR (CCl_4) δ 2.10 (t, 1, CH, J_{ab} = 1.5 Hz), 3.80 (s, d, 3, NH, NCH_2), 6.48 (d, 2, Ph), 7.00 (d, 2, Ph). *Disubstituted* aniline: NMR (CCl_4) δ 2.12 (t, 2, C–H), 4.00 (d, 4, CH_2N), 6.82 (d, 2, Ph), 7.05 (d, 2, Ph). A similar procedure yielded *N*-propargyl-4-chloroaniline.

N-[[[4-(Trifluoromethoxy)phenyl]propargylamino]-carbonyl]-2-chlorobenzamide (21). 2-Chlorobenzoyl isocyanate (0.083 mol) was added via syringe to a stirred solution of *N*-propargyl-4-(trifluoromethoxy)aniline (0.074 mol) in 20 mL of dry benzene. The mixture was stirred for 18 h at room temperature, the benzene removed under reduced pressure, and the resulting white crystals recrystallized twice from ethyl acetate–hexane to yield the *N*-propargyl derivative of BAY SIR 8514 (21, mp 134.5–134.6 °C) in 61% yield: NMR ($CDCl_3$) δ 2.20 (t, 1,

CH), 4.40 (d, 2, NCH_2), 7.40 (m, 8, Ph), 7.75 (br s, 1, NH). A similar procedure yielded *N*-[[(4-chlorophenyl)-propargylamino]carbonyl]-2,6-difluorobenzamide (5, *N*-propargyl derivative of diflubenzuron, mp 144–145.5 °C) in 56% yield. The propargyl derivatives (5 and 21, 100 mg of each) were reduced in the presence of 5 mg of Adams catalyst [Pt(IV) oxide, 40 mesh] in 10 mL of ethyl acetate by stirring under a blanket of D_2 delivered at slightly above atmospheric pressure with a gas buret. The resulting *N*-propyldiflubenzuron (6, mp 122–123 °C) and *N*-propyl-substituted BAY SIR 8514 (22, mp 112–114 °C) were chromatographically pure, and there was no evidence for halogen loss based upon the NMR aromatic splitting patterns or upon excess D_2 uptake.

Sulfenylated Diflubenzuron (7). The requisite sulfenyl chloride (0.05 mol) (Fahmy et al., 1978) was added to a vigorously stirred solution of 10 mmol of 4-chloroaniline in 25 mL of anhydrous ether at room temperature. The 4-chloroaniline hydrochloride begin to precipitate immediately, and after 2 h the turbid green solution was filtered through Celite and the ether removed under reduced pressure. The desired sulfenylated aniline accounted for 90% of the residual yellow oil when analyzed by NMR. The product was stable to purification by chromatography on Florisil and to preparative TLC: NMR (CCl_4) δ 0.8–1.8 (m, 10, H), 3.52 (q, 2, NCH_2), 4.10 (t, 2, OCH_2), 6.50 (s, 1, NH), 7.20 (dd, 4, Ph). The sulfenylated aniline was condensed with 2,6-difluorobenzoyl isocyanate as described earlier and the product purified by preparative TLC in hexane–ethyl acetate (3:2) to give a colorless oil in 65% yield: NMR ($CDCl_3$) δ 0.95 (t, 3, CH_3), 1.45 (m, 4, CH_2), 3.35 (q, 2, CH_2N), 4.20 (t, 2, OCH_2), 6.82 (t, 2, Ph), 7.20 (m, 5, Ph), 11.05 (br s, 1, NH). The other sulfenylated derivatives (8, 9, and 23) were prepared in an analogous manner. All compounds yielded the parent benzoylphenylurea when exposed to weak acid or water at pH 7.4, but they were stable in organic solvents containing a trace of pyridine. Compounds 7 and 9 were clearly less stable than the *N*-isopropyl carbamates 8 and 23.

Active Ester Synthesis. *N*-Hydroxysuccinimide (0.18 mmol) and the diflubenzuron *N*-carboxypropyl derivative (4) were dissolved in 3 mL of dry tetrahydrofuran and cooled to 5 °C. *N,N*-Dicyclohexylcarbodiimide (DCC, 0.4 mmol) was added in 800 μL of tetrahydrofuran to the vigorously stirred solution. After 0.5 h the ice bath was removed and the reaction allowed to stir for 20 h at room temperature. The reaction was stopped by the addition of 30% acetic acid (1 mL) and brine (0.5 mL), and the organic layer was removed. The aqueous phase was extracted with ether (3 mL, 2×), and the combined organic extracts were filtered through Celite and anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue purified by preparative TLC to give 85% yield of the diflubenzuron *N*-carboxypropyl active ester. The structure was confirmed by the presence of succinimide protons at δ 2.75 ($CDCl_3$), the conversion of the active ester to the parent butyrate by base treatment, and preparation of the tyramine derivative (Wing et al., 1978).

Bioassays. Several of the compounds synthesized were assayed for insect growth regulator activity. Twenty early last larval instar mosquito larvae (*Culex pipiens quinquefasciatus*) were placed in waxed paper cups containing 100 mL of tap water and 0.5 mL of 0.01% pyridine in acetone containing the test compound. Subsequently, 100 mg of a diet consisting of a mixture of 3 parts of ground Purina dog chow and 1 part of brewer's yeast was added. Alternatively, 10 last instar "white" housefly larvae (*Musca domestica*) that had partially cleared their guts were placed on filter paper in a 9 cm × 15 mm plastic Petri plate containing a second 3 cm × 10 mm Petri plate filled with water. The larvae were treated topically with the test compound in 0.5 μL of 0.01% pyridine in tetrahydrofuran. Finally, early day 3 (1 day prior to pupation) last instar larvae of the cabbage looper (*Trichoplusia ni*) were placed in 1-oz plastic pill cups containing 3 mL of a pinto bean based diet (Shorey and Hale, 1965) and treated topically with the test compound in acetone in pyridine. In each case the test insects were held at 24 °C on a 16:8 light:dark cycle and insects which failed to emerge as adults were scored as dead (Mumby et al., 1979). The dosage–mortality data were corrected for the minimal control mortality by using Abbott's formula (Abbott, 1925), and the biological activity of the experimental compound was compared to that of diflubenzuron or BAY SIR 8514 obtained in the same bioassay.

Antigen Preparation. *Active Ester Coupling Method.* The requisite protein (50 mg) was dissolved in 5 mL of water and the solutions titrated with 0.1 N sodium hydroxide to pH 8.5–8.8. The solutions were cooled in an ice bath and the appropriate active ester (0.028 mmol) was added dropwise in 5 mL of tetrahydrofuran–water (1:1). The solutions were stirred for 18 h at 4 °C and then dialyzed against four changes of 0.05 M ammonium bicarbonate followed by four changes of water, all at 4 °C. The proteins were then lyophilized and weighed into vials for storage under N_2 at −77 °C. All proteins were from Calbiochem and include bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin (OA), and rabbit serum albumin (RSA).

Water-Soluble Diimide Coupling Method. The requisite protein (50 mg) was dissolved in cold water (8 mL) and titrated to pH 6.5 with a pH meter. To each sample was added 25 mg of Morpho CDI [1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate] followed immediately by addition of 0.028 mmol of the appropriate free acid (4, 11, 15, 20, 25) in 2 mL of dimethylformamide–water (1:5). The reaction was allowed to proceed at room temperature for 18 h and then dialyzed against four changes of 0.05 M ammonium bicarbonate followed by four changes of water. The protein conjugates were then lyophilized and stored as above.

Immunization. Separate groups of New Zealand white rabbits were immunized with antigens in 7 mM phosphate buffer containing 0.15 M NaCl (phosphate-buffered saline, PBS) emulsified with an equal amount of complete Freund's adjuvant (Wing et al., 1978). A volume of 0.7–1 mL of antigen emulsion was injected at multiple intradermal and subcutaneous sites. Booster injections were then given at monthly intervals by using the same antigen emulsified in incomplete Freund's adjuvant. Blood was obtained from the rabbits' ear vein 1 week after the injection and the serum was separated by centrifugation and stored at −20 °C. By use of [^{14}C]diflubenzuron as the labeled antigen, anti-diflubenzuron antibody was generally demonstrated in rabbit plasma after 2 months of immunization.

Radioimmunoassay. The specificity of anti-diflubenzuron antibodies was first detected by radioimmunoassay using [^{14}C]diflubenzuron (Thompson-Hayward Chemical Co., 16 mCi/mmol ring-^{14}C) as the radioligand. The assays were performed in 6 × 50 mm glass tubes containing 200 μL of serum diluted with sodium phosphate buffer solution, I = 0.2 M, pH 7.4, and 0.01% NaN_3 containing 0.02% normal rabbit immunoglobulins. Radiolabeled [^{14}C]diflubenzuron was added to the diluted serum in 1 μL of tetrahydrofuran, incubated at 37 °C for 0.5 h

and at 4 °C for 12 h. Unbound radioligand was precipitated by the addition of 100 μL of a charcoal dextran suspension (1% Mallinckrodt activated "Norit A" charcoal and 0.25% Pharmacia Dextran T70 in phosphate-buffered saline) followed by centrifugation (4 °C, 20 min, 1000g). Radioactivity in the supernatant was determined by liquid scintillation counting of a 100-μL aliquot in a cocktail consisting of 2 parts of OCS (a xylene-based scintillation solution, Amersham/Searle) and 1 part of Triton X-100.

Immunodiffusion. Double diffusion in agar was performed by the method of Ouchterlony (1968). One percent agarose gels (GIBCO) in PBS, pH 7.4, containing 0.05% NaN_3 were prepared at a depth of 2 mm on Petri dishes (35 × 10 mm). Wells were 3 mm in diameter with a center-to-center distance of 9 mm; 20-μL samples at protein concentrations of 0.25–1.25 mg/mL were placed in each well. Diffusion was carried out at room temperature for 16 h. Afterward, the plates were washed overnight in PBS, pH 7.7, followed by distilled H_2O, dried, and stained in 0.01% Coomassie blue.

Immunoelectrophoresis. Immunoelectrophoresis was performed following standard procedures (Scheidegger, 1955). Microslides were coated with 3 mL of 1% agarose gel in Tris–barbital buffer, pH 8.8. The application pattern in the gel consisted of two sample holes (diameter 1 mm) and one antibody trough; the distance between the holes and the trough was 6 mm.

RESULTS AND DISCUSSION

Synthesis. The benzoylphenylureas and related compounds promise to be of increasing importance; thus one purpose of this study was to illustrate several ways in which haptens for these compounds could be prepared. In general an antibody population will show the greatest specificity for that portion of the hapten molecule distal from the carrier protein. As illustrated by the synthetic approaches in Figures 1–3, the resulting antibodies should show the greatest specificity for the benzamide portion of the molecule and thus distinguish diflubenzuron and BAY SIR 8514.

Several of the attempted routes of hapten synthesis involved the direct alkylation or acetylation of the carbonylamide nitrogen. In spite of attempting numerous reaction conditions and reagents including alkyl halides, acyl halides, and anhydrides, we always obtained low yields except in the case of ethyl bromoacetate, which cyclized to yield compound 16 (Figure 3). A more successful approach involved derivatization of the aniline nitrogen followed by reaction with the appropriate isocyanate (Figure 1). The sulfenyl bond was examined as a potential ligand, but in each case the sulfenylated ureas (7–9, 23) proved to be unstable at neutral pH, rapidly yielding the parent urea. When *p*-chloroaniline was exposed to ethyl bromoacetate or ethyl 4-bromobutyrate and the resulting *N*-substituted aniline reacted with the appropriate isocyanate, the resulting *N*-(ethoxycarbonyl)methyl or *N*-(ethoxycarbonyl)propyl derivatives of diflubenzuron and BAY SIR 8514 were obtained in good yield (2, 3, 19). Cyclization followed cleavage of the ethyl ester of the diflubenzuron *N*-(ethoxycarbonyl)methyl derivative to yield 17. Analogous cyclization reactions were described earlier for the propionate and acetate derivatives of the 2,6-dichloro derivative (Wellinga et al., 1973a), but the base-catalyzed hydrolysis of the *N*-(ethoxycarbonyl)propyl derivatives (3, 19) to diflubenzuron and BAY SIR 8514 *N*-carboxypropyl derivatives (4, 20) proceeded in high yield.

The 3′-phenol of diflubenzuron rapidly reacted with ethyl bromoacetate to yield 14. The ethyl ester was subsequently hydrolyzed with base to the free acid 15. This pathway illustrates that compounds synthesized as potential metabolites may provide convenient handles for conjugation. Analogous small scale reactions with the 2′-phenol of diflubenzuron ultimately led to cyclization with the aniline nitrogen following hydrolysis.

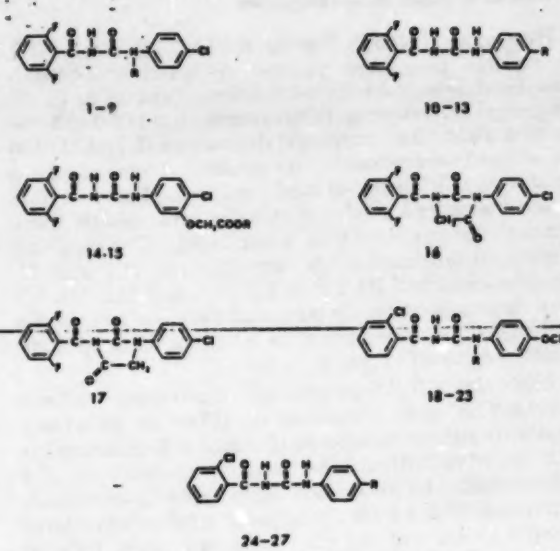

Figure 3. Compounds synthesized as potential haptens and radioligands for subsequent immunoassay of the insect growth regulators diflubenzuron (1) and BAY SIR 8514 (18). (1) R = H; (2) R = $CH_2COOC_2H_5$; (3) R = $(CH_2)_3COOC_2H_5$; (4) R = $(CH_2)_3COOH$; (5) R = $CH_2C{\equiv}CH$; (6) R = $CH_2CD_2CD_2H$; (7) R = $SN(C_2H_5)COOC_4H_9$; (8) R = $SN(i\text{-}C_3H_7)COOC_3H_7$; (9) R = $SN(C_3H_7)COOCH_3$; (10) R = CH_2COOCH_3; (11) R = CH_2COOH; (12) R = NO_2; (13) R = NH_2; (14) R = C_2H_5; (15) R = H; (18) R = H; (19) R = $(CH_2)_2COOCH_3$; (20) R = $(CH_2)_2COOH$; (21) R = $CH_2C{\equiv}CH$; (22) R = $CH_2CD_2CD_2H$; (23) R = $SN(i\text{-}C_3H_7)COOC_3H_7$; (24) R = CH_2COOCH_3; (25) R = CH_2COOH; (26) R = NO_2; (27) R = NH_2.

Two additional handles were placed in the 4′ position of diflubenzuron and BAY SIR 8514 by reaction of an appropriately substituted aniline with 2,6-difluoro- or 2-chlorobenzoyl isocyanate. The ethyl 4′-phenylacetates (10, 24) were hydrolyzed to the corresponding phenylacetic acids (11, 25), and the 4′-nitro derivatives (12, 26) were reduced with hydrogen over palladium/charcoal to the 4′-anilines (13, 27). Both carboxylic acids and amines can be coupled to proteins by a variety of methods. These compounds are very similar to analogues of the benzoylphenylureas previously synthesized for bioassay (Wellinga et al., 1973a,b; Yu and Kuhr, 1976) and illustrate that in some cases the pesticide itself or a previously synthesized analogue can be used as a hapten for conjugation.

The theoretical sensitivity of a radioimmunoassay is generally proportional to the square root of the specific activity of the radioligand used. As shown in this report, the low specific activity ^{14}C-labeled diflubenzuron used was adequate for a determination of antibody titers, but it was not suitable for a highly sensitive assay. Two approaches were thus taken to facilitate subsequent preparation of a high specific activity ligand. In one approach the tyramine derivative of the diflubenzuron *N*-carboxypropyl derivative was prepared from the corresponding active ester. The tyramine derivative serves two purposes. First, tyramine can be coupled to a hapten under the same conditions as a protein; then the suspect tyramine-coupled hapten can be extracted and compared chromatographically with the authentic derivative. This procedure tests the effectiveness

of the protein coupling techniques. Second, tyramine, the related Bolton–Hunter reagent (Bolton and Hunter, 1973), and numerous amino acids are commercially available with high specific activity tritium radiolabels, and in some cases they can be readily iodinated. As recently illustrated by the work of Wing and Hammock (1979) on *S*-bioallethrin and Fatori and Hunter (1980) on paraquat, such radiolabels can yield sensitive immunoassays.

In the second approach, *N*-propargyl derivatives of diflubenzuron (5) and BAY SIR 8514 (21) were prepared and catalytically reduced to yield the corresponding D_4 compounds (6, 22). The reaction conditions were optimized and the products characterized so that propargyl derivatives could subsequently be reduced with tritium gas, yielding inexpensive radioligands of very high specific activity. As previously discussed (Parker, 1976; Hammock and Mumma, 1980) an intrinsic radiolabel is not necessary for a radioimmunoassay, and the two approaches described above illustrate inexpensive approaches to obtaining such radiolabels. In fact, if the antibody has a higher affinity for the molecule of interest than for the radioligand, the theoretical assay sensitivity will increase.

Biological Activity. Diflubenzuron *N*-(ethoxycarbonyl)methyl derivative (2) was essentially nontoxic at 10–100 times the LD_{50} of diflubenzuron to the three insects tested. Similar results were obtained with the diflubenzuron *N*-(ethoxycarbonyl)propyl derivative (3) except that it was only 10-fold less toxic than diflubenzuron to *T. ni*. The greatly reduced insecticidal action of the *N*-(ethoxycarbonyl)methyl and *N*-(ethoxycarbonyl)propyl derivatives of diflubenzuron is not surprising based on previous studies (Wellinga et al., 1973a,b). The sulfenylated derivatives were rapidly hydrolyzed to yield the parent diflubenzuron under even very slightly acidic conditions, although they were very stable in the presence of trace amounts of pyridine. Thus, the insecticidal activity of these compounds is probably due to the regeneration of the parent compound as is the case for sulfenyl derivatives of carbamate insecticides. Unlike some insecticidal carbamates (Fahmy et al., 1978), the insecticidal activities of the sulfenylated carbamates of diflubenzuron and BAY SIR 8514 are not dramatically increased. However, the physical state of the IGRs is changed dramatically. These benzoylphenylureas are crystalline solids that are only slightly soluble in solvents such as tetrahydrofuran or dimethylformamide and insoluble in aliphatic and aromatic solvents. However, the sulfenylated derivatives are oils that are totally miscible with hexane and most other organic solvents and thus could be easily formulated. Problems associated with current benzoylphenylurea formulations could possibly be avoided with such derivatives (Maas et al., 1980). The toxicity of the sulfenylated derivatives (7, 8, 9, 23) was similar or slightly less than the toxicity of the parent compound on the three insects examined.

Several benzoylphenylureas have been reported to inhibit chitin synthesis in in vitro systems in the nano- or picomolar range, and one hypothesis of action is that such compounds block proteases responsible for the activation of chitinase zymogens (Maas et al., 1980; Leighton et al., 1981). The structure of diflubenzuron certainly supports the idea that it may act as a quasi-substrate or a transition state mimic of such a protease. Since for affinity chromatography inhibitors with I_{50}'s in the milli- or micromolar regions are usually the most effective, the synthetic approaches described here may be very useful for isolating the protease or other targets of benzoylphenylurea action.

Development and Analysis of Antisera. Following careful hydrolysis of the methyl or ethyl esters to yield haptens with free carboxylic acid functionalities (4, 11, 15, 20, 25), the haptens were then coupled to several proteins by using one or more of the two coupling methods previously described. Both methods yielded an amide linkage with 2–5 mol of hapten/50 000 daltons of protein. This loading was initially estimated by analysis of the free amines before and after conjugation and subsequently by enzyme-linked immunosorbent assay (ELISA). Thus, rabbits A and B were immunized with *N*-(carboxypropyl)diflubenzuron–KLH prepared by the active ester method. All other antigens were prepared by the water-soluble carbodiimide method and rabbits were inoculated as follows: rabbits C and D [*N*-(carboxypropyl)diflubenzuron–BSA], rabbit E [*N*-(carboxypropyl)diflubenzuron–KLH], and rabbits F and G (diflubenzuron phenoxyacetic acid–KLH).

Figure 4 summarizes the results from screening the sera of the seven rabbits for anti-diflubenzuron antibodies by RIA using [^{14}C]diflubenzuron as a tracer. The specificity of the RIA is shown by the near background radioactivity bound by the nonimmune serum. Although all rabbits

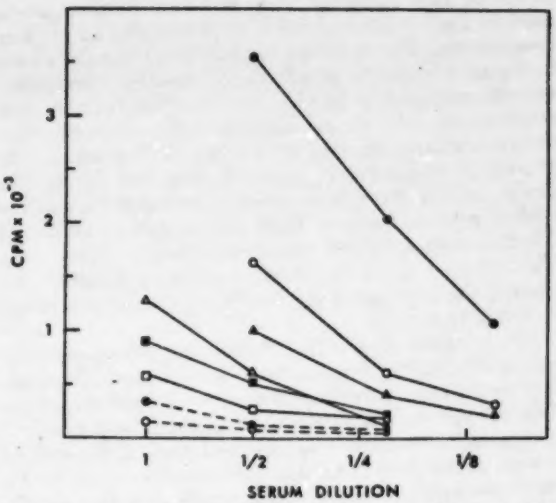

Figure 4. Radioimmunoassay of serum from rabbits A–G at several dilutions. The counts (cpm) bound by serum are plotted against the serum concentration used. Each tube contained 4900 cpm of [^{14}C]diflubenzuron (16 mCi/mmol). Lines represent sera from rabbits A (△—△), B (■—■), C (●—●), D (○—○), E (▲—▲), F (□—□), and G (●- - -●) and nonimmune serum (○—○).

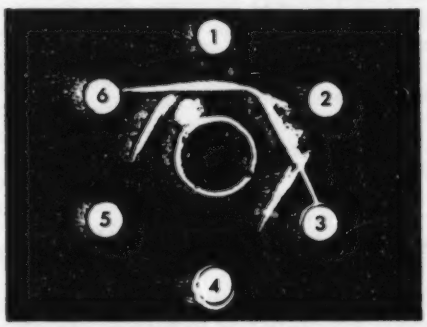

Figure 5. Ouchterlony plate of antibodies from rabbit C against different antigens. Center well: anti-*N*-(carboxypropyl)diflubenzuron–BSA antibodies (undiluted). Outer well: (1) *N*-(carboxypropyl)diflubenzuron–BSA, 1 mg/mL; (2) BSA, 1 mg/mL; (3) *N*-(carboxypropyl)diflubenzuron–KLH, 0.5 mg/mL; (4) KLH, 1.25 mg/mL; (5) OA, 1 mg/mL; (6) *N*-(carboxypropyl)diflubenzuron–OA, 0.25 mg/mL.

produced anti-diflubenzuron antibodies, only rabbits C–E produced high antibody titers which were subsequently used for the residue analysis of diflubenzuron and related compounds. The relative antibody titers were as shown in Figure 4 when the sera from rabbits A–G were subsequently analyzed by ELISA, but the absolute titers were much higher. Specific anti-diflubenzuron antibodies were detected within 2 months after immunization, and a total of over 20 immunizations were carried out during this study. It was found that repeated immunization of the rabbits with the same antigen did not have a profound effect on their specific antibody production. Thus, those rabbits that had high antibody titers after 2 months remained the high antibody producers during the entire course of this work.

The presence of specific anti-benzoylphenylurea antibodies was further demonstrated by numerous Ouchterlony gel diffusion experiments as shown for diflubenzuron *N*-carboxypropyl derivative and antiserum from rabbit C in Figure 5. Antibodies against both the hapten and its protein carrier were present in the antiserum as shown by a spur projecting from diflubenzuron *N*-(carboxypropyl)-diflubenzuron–BSA (well 1) extending over the precipitation band to normal BSA (well 2). Moreover, when the same hapten was coupled to KLH as a carrier, a precipitation band was observed (well 3) that crossed the BSA precipitation band (well 2) as expected. Similarly, a clear precipitation band between the hapten coupled to OA (well 6) and the antiserum was observed. The BSA–hapten precipitation band (well 1) clearly demonstrates a spur that crosses the band from well 6. The presence of an antigen component attributable to the hapten was also demonstrated by the lack of reaction between the antiserum and either KLH or OA (wells 4 and 5, respectively). Immunoelectrophoretic analysis (results not shown) revealed that all the modified antigens (BSA, KLH, OA) had more anodal mobility than the corresponding unmodified proteins. These results are not surprising since the hapten was coupled to the carrier largely via the lysyl moieties of the protein molecules.

Conclusion. As amply documented by van Daalen et al. (1972) and in a variety of subsequent publications (Maas et al., 1980), the benzoylphenylurea IGRs offer numerous advantages as insect control agents. However, the expense and tedium of the classical analytical procedures for these compounds is undoubtedly one factor retarding their expanded registration. For such compounds the speed and simplicity of immunochemical analysis may offer numerous advantages (Hammock and Mumma, 1980). The simple, high-yield synthetic approaches presented here for the IGRs diflubenzuron and BAY SIR 8514 should be generally applicable to the synthesis of haptens for a variety of structurally related IGRs and other pesticides. The development of sensitive, specific immunoassays for the above IGRs and the use of these assays in residue analysis will be discussed in the following paper (Wie and Hammock, 1982).

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